The Histone H2A Deubiquitinase USP16 Interacts with HERC2 and Fine-tunes Cellular Response to DNA Damage*

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Background: Protein factors that negatively regulate DNA damage-induced ubiquitin foci formation are less known.

Results: USP16 interacts with HERC2, negatively regulates DNA damage-induced ubiquitin foci formation, and is required for termination of the ubiquitin signal.

Conclusion: USP16 functions as a negative factor of DNA damage-induced ubiquitin foci formation.

Significance: Understanding how ubiquitin signal is precisely controlled will help understand the mechanism for DNA damage repair.

Histone ubiquitination at DNA double strand breaks facilitates the recruitment of downstream repair proteins; however, how the ubiquitination is dynamically regulated during repair and terminated after repair is not well understood. Here we report that the histone H2A deubiquitase USP16 interacts with HERC2, fine-tunes the ubiquitin signal during repair, and importantly, is required for terminating the ubiquitination signal after repair. HERC2 interacts with the coiled-coil domain of USP16 through its C-terminal HECT domain. HERC2 knockdown affects the levels of ubiquitinated H2A through the action of USP16. In response to DNA damage, USP16 levels increase, and this increase is dependent on HERC2. Increased USP16 serves as a negative regulator for DNA damage-induced ubiquitin foci formation and affects downstream factor recruitment and DNA damage response. The functional significance of USP16 is further manifested in human Down syndrome patient cells, which contain three copies of USP16 genes and have altered cellular response to DNA damage. Finally, we demonstrated that USP16 could deubiquitinate both H2A Lys-119 and H2A Lys-15 ubiquitination in vitro. Therefore, this study identifies USP16 as a critical regulator of DNA damage response and H2A Lys-15 ubiquitination as a potential target of USP16.

Covalent modifications of histones, including acetylation, methylation, phosphorylation, and ubiquitination, are important regulators of chromatin-based nuclear processes including transcription and DNA damage repair (1, 2). These modifications affect chromatin structure and function directly or provide a platform for the recruitment and exclusion of protein factors to the chromatin template. Ubiquitination of the histone H2A Lys-119 residue, the first identified ubiquitin conjugate, is primarily catalyzed by polycomb repressive complex 1 (PRC1)3 and has been linked to gene repression (3, 4). However, recent studies have revealed that histone H2A is also ubiquitinated at another site(s) than Lys-119, which plays important roles in physiological processes such as cellular response to DNA damage. Upon DNA damage, ubiquitin conjugates are transiently enriched at DNA damage foci (5–7). Within these ubiquitin conjugates, RNF168-catalyzed H2A Lys-13/15 ubiquitination is required for the efficient recruitment of the downstream repair protein, 53BP1 (8–11). H2A Lys-13/15 ubiquitination recruits 53BP1 to DNA damage foci through the recently identified 53BP1 ubiquitin interaction motif (residues 1604–1631) (12). However, PRC1-mediated H2A Lys-119 ubiquitination may also play an important role during cellular response to DNA damage, as subunits of PRC1 including Ring2 and Bmi1 are also recruited to DNA damage foci, and knockdown of Ring2 and Bmi1 impairs DNA damage-induced ubiquitin foci formation (7, 13–15). Currently, it remains unclear which H2A ubiquitin conjugate(s) is present at DNA-damaged ubiquitin foci and how these ubiquitinations coordinate during DNA damage repair. Nonetheless, the ubiquitination pathway appears to be critical for normal cellular function, as mutation of RNF168 causes RIDDLE (radiosensitivity, immunodeficiency, dysmorphic features, and learning difficulties) syndrome in humans (16).

The enrichment of ubiquitin conjugates at DNA damage foci is a dynamic process; ubiquitin levels increase quickly in response to DNA damage, peak at 1–4 h, and then gradually decrease to normal levels ~24 h after DNA damage (5–7). The dynamic change in ubiquitin foci reflects the kinetics of DNA damage repair. Supporting this idea, perturbation of ubiquitination dynamics by knockdown of the ovarian tumor (OTU) domain-containing protein OTUB1 results in persistent ubiquitination after exposure to ionizing radiation, impairs repair foci formation downstream of RNF168, and causes defects in damaged DNA repair (17). However, OTUB1 suppresses

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RNF168-dependent ubiquitination independently of its deubiquitinase activity by directly binding and inhibiting UBC13 (also known as UBE2N), the cognate E2 enzyme for RNF168. BRCC36, USP3, and USP44, three deubiquitinases, have also been shown to antagonize DNA damage-induced H2A ubiquitination (18–20). Among these deubiquitinases, USP3 could deubiquitinate H2A Lys-13/15 ubiquitination directly when overexpressed; however, it remains unclear whether endogenous USP3 plays a role in this process (21). It is also not clear whether other enzymes can also catalyze H2A Lys-119 or Lys-15 deubiquitination and play a role during DNA damage repair. Moreover, once damaged DNA is repaired, the enzyme responsible for terminating the ubiquitin signal is not known.

Here, we identify USP16 as a negative regulator of DNA damage-induced ubiquitin foci and damaged DNA repair process. Importantly, USP16 is required for termination of ubiquitin signals after DNA damage repair. HERC2 interacts with USP16 and regulates USP16 protein levels. Upon DNA damage, USP16 levels increase quickly, and this increase requires HERC2. The increased USP16 levels negatively regulate DNA damage-induced ubiquitin foci and are required for the proper termination of the ubiquitin signal. The functional significance of USP16 in DNA damage repair is further manifested in human Down syndrome cells, which contain three copies of USP16. Down syndrome cells, which contain three copies of USP16, show increased levels of ubiquitin foci and are required for the proper termination of ubiquitin signals after DNA damage repair. HERC2 interacts with USP16 and regulates USP16 protein levels. Upon DNA damage, USP16 levels increase quickly, and this increase requires HERC2. The increased USP16 levels negatively regulate DNA damage-induced ubiquitin foci and are required for the proper termination of the ubiquitin signal. The functional significance of USP16 in DNA damage repair is further manifested in human Down syndrome cells, which contain three copies of USP16 genes and exhibit altered cellular damage response. Therefore, these studies identify USP16 as a negative regulator of the DNA damage response which fine-tunes DNA damage-induced histone ubiquitin foci formation.

EXPERIMENTAL PROCEDURES

Plasmids—Plasmids used for expression of H2A K13R/K15R, K118R/K119R, and K13R/K15R/K118R/K119R were kindly provided by Dr. Sixma (11). Plasmids used for expression of HERC2 fragment were kindly provided by Dr. Miall (22). Myc-RNF8 was kindly provided by Dr. Junjie Chen, and GFP-USP16 was provided by Dr. Xiaochen Yu. Plasmids for expression of USP16 fragments were constructed by ligating PCR products of the USP16 cDNA into pcMV-3Tag-2A plasmid and confirmed by DNA sequencing. USP16 expression vector was described in previous publication (23). I-Scel was a kind gift from Dr. Eddy Yang from the University of Alabama at Birmingham.

Cell Culture, Transfection, DNA Damage, and Clonogenic Assay—The human osteosarcoma cell line U2OS and SV40 T antigen transformed human embryonic kidney 293 cell line 293T were purchased from ATCC (Manassas, VA) and cultured in DMEM (HyClone, South Logan, UT) supplemented with 10% FBS (HyClone) and 1% ampicillin-streptomycin (HyClone). H1299 dA3–1 cells were kindly provided by Dr. Takashi Kohno, and the non-homologous end joining (NHEJ) assay was performed as described (24). The HEK293D/DRGFP cell line was kindly provided by Dr. Fen Xia, and the homologous recombination (HR) assay was performed as described (25). DNA-PKcs inhibitor NU7026 (50 μM) was added to cells when I-Scel transfection was performed. siRNAs against human HERC2 were purchased from Sigma, and the sequences are 5′-caacagguagcagga and 5′-gaacagcagcagga. siRNAs against USP16 were described in a previous publication (23). siRNA transfection was performed with Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. Plasmid transfection was performed with Effectene (Qiagen) following the manufacturer’s instructions. To induce DNA damage, cells were exposed to γ-radiation (by GC-40 Gamma Cell Irradiator) and fixed or harvested at the indicated time points for immunofluorescent staining or Western blot assay. For the clonogenic assay, cells in 6-well plates were transfected with the indicated siRNA and plated, exposed to γ-radiation, and cultured for 2–3 weeks before fixing with 6% glutaraldehyde and staining with 0.5% crystal violet.

Immunoprecipitation and Immunofluorescent Staining—For immunoprecipitation, affinity-purified USP16 antibody was cross-linked to protein A-agarose beads as described (23). Whole cell extracts were prepared by incubating control (see Fig. 1) and transfected 293T cells (FLAG-HERC2 fragments and Myc-USP16 fragments) with TNM buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, protease inhibitors) at 4 °C for 30 min with rotation. After centrifugation, whole cell extracts were incubated with cross-linked USP16 antibody, anti-FLAG M2-agarose (Sigma), or anti-Myc-agarose (Sigma) at 4 °C for 2–4 h. After washing with BC50 three times and BC500 two times, proteins bound to beads were eluted with glycine (for USP16 antibody) or the corresponding peptides (customer synthesis) and used for Western blot assay with specific antibodies.

For detection of ubiquitinated H2A, transfected cells were directly dissolved into denaturing buffer (20 mM Tris, pH 7.4, 50 mM NaCl, 0.5% Nonidet P-40, 0.5% deoxycholate, 0.5% SDS, 1 mM EDTA, and protease inhibitors) by sonication. After centrifugation, cell lysates were used for Western blot assay with HRP-conjugated anti-FLAG antibody (for wild type H2A and Lys-13/15-mutated H2A). For H2A K13R/K15R and four lysine mutant H2A mutants, cell lysates were subjected to anti-FLAG M2-agarose (Sigma) immunoprecipitation. After washing with denaturing buffer extensively (5 min, 5 times each), FLAG-H2A was eluted by FLAG peptide in denaturing buffer. The eluate was then used for Western blot assay. For USP16 ubiquitination assay, MG132 (10 μM, final concentration) was added to cell culture 6 h before harvesting the cells and was included in all subsequent buffers.

For immunofluorescence staining, U2OS cells grown on coverslips were fixed with 4% formaldehyde and permeabilized with 0.5% Triton X-100 followed by blocking in 3% BSA for 15 min. For staining with FK2 antibody, cells were pretreated with nuclear extraction buffer (20 mM HEPES, pH 7.5, 20 mM NaCl, 5 mM MgCl₂, and 1 mM dithiothreitol (DTT), 0.5% IGEPAL) for 20 min on ice before fixation. Cells were then incubated with anti-FK2, or anti-ub-H2A (E6C5) or 53BP1 or anti-Myc or anti-FLAG antibodies at room temperature for 2 h. After washing cells with PBS for 5 min four times, cells were then incubated with FITC-conjugated secondary antibody at room temperature for 1 h. The coverslips were then washed, counter-stained with DAPI, and mounted in 0.2% n-propyl galate. For GFP-RNF168, cells grown on coverslips were transfected, grown for 24 h, and then fixed, counter stained with DAPI, and mounted in 0.2% n-propyl galate. Cells were examined under Olympus fluorescence microscopy. For counting the DNA damage-induced
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Nucleosome Preparation and Histone Deubiquitination Assay—Mononucleosomes and core histones were purified from 293T cells expressing H2A K13R/K15R or K118R/K119R following a small scale protocol developed in our laboratory (26). Briefly, cells from 2–3 10-cm plates were harvested. Nuclei were purified by incubating cells with Buffer A (0.2 M sucrose, 60 mM KCl, 10 mM NaCl, 15 mM MES, pH 6.5, 5 mM MgCl2, 1 mM CaCl2, 0.5% Triton X-100, 1 mM DTT, 0.2 mM PMSF, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 1 μg/ml aprotinin) for 15 min at 4 °C. After equilibrating nuclei with micrococcal nuclease digestion buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 2 mM MgCl2, 0.3 M sucrose, 1 mM DTT, 0.2 mM PMSF, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 1 μg/ml aprotinin, 5 mM CaCl2), nuclei were digested by 5 μl of micrococcal nuclease (200 units/μl, Sigma) for 20 min at 37 °C to give a complete digestion. Mononucleosomes were extracted from digested nuclei by incubation with nucleosome extraction buffer (20 mM Tris HCl, pH 7.9, 10 mM EDTA, 0.5 M NaCl, protease inhibitors). After dialyzing against histone storage buffer (10 mM Hepes-KOH pH 7.5, 1 mM EDTA, 10 mM KCl, 10% glycerol, 0.2 mM PMSF) for 2 h, mononucleosomes were used for deubiquitination reaction or for core histone preparation. For core histone preparation, dialyzed mononucleosomes were incubated with 200 μl of hydroxyapatite beads (Bio-Rad, 130-0151) at 4 °C for 20 min. After washing with NP300 and BP500 (40 mM Na2HPO4/NaH2PO4, pH 6.8, 0.3 M or 0.5 M NaCl, 1 mM DTT, and 0.2 mM PMSF), histones were eluted by NP2500. After dialysis against histone storage buffer, core histones were used for deubiquitination assay. Histone deubiquitination reactions were performed as described (23). Briefly, equal amounts of mononucleosomes or core histones were incubated with 0.5 μg of USP16 protein (measured by Bradford methods) purified from sf9 cells (by anti-FLAG immunoprecipitation and then gel filtration) in deubiquitination reaction buffer (100 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1 mM PMSF, 1 mM DTT, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A) at 37 °C for 1 h. The reaction was terminated by the addition of SDS-PAGE sample loading buffer, and proteins were resolved on SDS-PAGE and blotted with the anti-FLAG antibody.

RESULTS

The Histone H2A Deubiquitinating USP16 Interacts with Endogenous HECT and RLD Domain-containing Ubiquitin E3 Ligase HERC2—Our previous purification of USP16 from HeLa cells did not identify any interacting proteins; however, the harsh conditions during purification could have disrupted protein-protein interactions (23). Consistent with this notion, retrovirus-mediated overexpression of FLAG-HA-USP16 in 293T cells combined with LC-MS/MS analysis identified three potential USP16 interacting proteins: HERC2 (HECT and RCC1-like domain-containing protein 2), HistI1H2B1L (histone H2B type 1-L), and DBT (lipoamide acyltransferase component of branched-chain α-keto acid dehydrogenase complex, mitochondrial precursor) (27). Of these three polypeptides, HERC2 has been shown to interact with RNF8 and be involved in cellular response to DNA damage, a process where histone H2A ubiquitination plays a role (22). Therefore, we first investigated whether USP16 and HERC2 interact under non-overexpression conditions. For this purpose we performed co-immunoprecipitation experiments using the USP16 antibody. As shown in Fig. 1A, USP16 antibody, but not control IgG, co-immunoprecipitated HERC2 (compare lane 2 with 3), confirming that HERC2 and USP16 interact endogenously. To determine which region(s) of HERC2 is involved in the interaction, we transfected FLAG-tagged HERC2 fragments (22) into 293T cells and performed co-immunoprecipitation with anti-FLAG antibody. As shown in Fig. 1B, only the HECT domain-containing C-terminal region of HERC2 (aa 4421–4834) co-immunoprecipitated USP16 (compare lane 7 with lanes 1–6). We noticed that the same region is also responsible for interaction with RNF8, raising the possibility of interplay between USP16 and RNF8 binding to HERC2 (22). To investigate this possibility, we transfected both HERC2 F6 and Myc-RNF8 into U2OS cells and performed immunoprecipitation assays. As shown in Fig. 1C, USP16 antibody could immunoprecipitate both HERC2 and RNF8, suggesting that there is a three-component complex formation (lane 3). Consistently, we discovered that myc-RNF8 could also co-immunoprecipitate both HERC2 and USP16 (Fig. 1C, lane 4). Together, these data suggest that USP16, HERC2, and RNF8 form a complex and that HERC2 might serve as a platform to recruit both RNF8 and USP16 during DNA damage response (22). Although the same region of HERC2 (F6) is involved in the interaction, specific sub-regions or sub-domains might be involved in the interaction with USP16 and RNF8, respectively. Alternatively, oligomerization may allow simultaneous interaction with both proteins (22). Based on these data and previous studies (22), it appears that HERC2 functions as a platform to assemble factors regulating DNA damage response.

Previous studies have also revealed that DNA damage (ionizing radiation) stimulates the association between RNF8 and HERC2 (22). To determine whether ionizing radiation also affects the interactions between USP16 and HERC2, we performed co-immunoprecipitation on cell extracts after ionizing radiation. As shown in Fig. 1D, ionizing radiation results in an increase of the amount of USP16 (Input, sixth panel, compare lanes 2 and 3 with 1). Consistently, we also observed increased amounts of both USP16 and HERC2 immunoprecipitated by USP16 antibody (Fig. 1D, top two panels). To distinguish whether there is indeed an increased interaction between USP16 and HERC2, we normalized the amounts of sample loading based on USP16. Notably, we observed an increased amount of HERC2 immunoprecipitated by equal amount of USP16 (Fig. 1D, third and fourth panels, compare lanes 2 and 3 with 1). Therefore, ionizing radiation enhances the interaction between USP16 and HERC2, which may facilitate the recruitment of USP16 under DNA damage conditions.

To determine which region of USP16 is involved in the interaction, we transfected Myc-tagged USP16 fragments and FLAG-HERC2 F6 into 293T cells and performed immunoprecipitation experiments with anti-FLAG antibody. As shown in Fig. 1E, the N-terminal region of USP16 (aa 136–185), which contains the first coiled-coil domain, is required for USP16 interaction with HERC2 (lane 3). The critical role
of the first coiled-coil domain was further demonstrated by the finding that USP16 mutant, which lacks the first coiled-coil domain, fails to immunoprecipitate HERC2 (Fig. 1F, compare lane 3 with 2). Therefore, our studies confirmed that USP16 interacts with HERC2 under non-overexpression conditions and further identified that the C terminus of HERC2 and the first coiled-coil domain of USP16 are involved in the interaction.
HERC2 Regulates USP16 and H2A Ubiquitination Levels—Because HERC2 interacts with USP16, we investigated how HERC2 regulates USP16 function. As shown in Fig. 2A, HERC2 siRNA transfection results in a significant reduction of HERC2 levels (top panel), and surprisingly it also reduces USP16 protein level (second panel). Due to the unavailability of HERC2 cDNA, the specificity of siRNA was validated with different target sequences and also using the HERC2 inducible knockdown cell lines (data not shown). Interestingly, HERC2 knockdown also results in a concomitant increase of H2A ubiquitination levels, which was detected by the E6C5 anti-ub-H2A monoclonal antibody (Fig. 2A, fourth panel), raising the possibility that HERC2 regulates H2A ubiquitination levels through USP16. However, because HERC2 also affects the levels of RNF168, a histone H2A ubiquitin ligase involved in DNA damage (22), HERC2 could possibly regulate H2A ubiquitination through RNF168. To distinguish these two possibilities, we investigated whether USP16 could rescue HERC2 knockdown-

**FIGURE 2.** HERC2 regulates USP16 and histone H2A ubiquitination levels. A, HERC2 knockdown results in a decrease of USP16 and an increase of H2A ubiquitination level. Western blot assay of cells transfected with control scrambled siRNA and siRNA against HERC2. GAPDH and total histone H2A are used as loading controls. Antibodies used are labeled on the left side of the panel. B, HERC2 knockdown affects H2A ubiquitination level at least in part through USP16. A Western blot assay of control and HERC2 knockdown cells transfected with constructs as indicated above is shown. Transfection of USP16 abolished an HERC2 knockdown-induced increase of H2A ubiquitination levels. GAPDH and total histone H2A were used as loading controls. Antibodies used are labeled on the left side of the panel. C, DNA damage induces dynamic changes of USP16 and H2A ubiquitination levels. A Western blot assay of U2OS cells at different time points after 3 gray ionizing irradiation. GAPDH and total histone H2A are used as loading controls. Antibodies used are labeled at the left side of the panels. D, quantification of dynamic changes of USP16 protein level and ub-H2A level after DNA damage. Image J software is used to quantify the intensity of USP16 and ubiquitinated H2A signal in panel C. The levels at time 0 were arbitrarily set as 1. E, HERC2 is required for the dynamic changes of USP16 and H2A ubiquitination levels in response to DNA damage. A Western blot assay of control and HERC2 knockdown cells at different time points after DNA damage is shown. The increases of USP16 and ub-H2A in response to DNA damage are dramatically reduced in HERC2 knockdown cells. Note: to see the signal at lane 6, the films were exposed at different times.
induced H2A ubiquitination level change. As shown in Fig. 2B, USP16 was expressed at similar levels in control and HERC2 knockdown cells (second and third panels, compared lane 3 with 4). However, transfection of USP16 completely abolished a HERC2 knockdown-induced increase of H2A ubiquitination levels (Fig. 2B, fourth panel, compare lanes 3 and 4 with 1 and 2). These data suggest that HERC2 regulates H2A ubiquitination levels at least in part through regulation of USP16 protein levels.

HERC2 has been shown to interact with RNF8 and coordinate DNA damage-induced histone ubiquitination events (22). To determine whether USP16 is also involved in cellular response to DNA damage, we measured the changes of USP16 levels after DNA damage. We found that USP16 levels underwent a dynamic change after irradiation and that USP16 increased dramatically in the first several hours and then gradually decreased to normal levels (Fig. 2C, top panel, compare lane 2–5 to 1; also see Fig. 2D for quantification). The levels of H2A ubiquitination also display a dynamic change in response to DNA damage, declining slightly in the first hour, peaking between 2 and 4 h, and then decreasing gradually to their original level (Fig. 2C, second panel, compare lanes 2–5 with lane 1; see also Fig. 2D for quantification). Surprisingly, the changes in H2A ubiquitination levels are not inversely correlated with USP16 protein levels, as predicted from previous studies (23). Similar changes of USP16 and H2A ubiquitination levels were also observed at different doses of ionizing radiation and different cell types (data not shown), indicating that the increase of both ub-H2A and USP16 levels might be a general feature of cellular DNA damage response. The increase of both USP16 and H2A ubiquitination after DNA damage suggests that USP16 is likely to fine-tune ubH2A levels during cellular response to DNA damage (see below for further information).

To determine whether the dynamic change of USP16 after DNA damage is controlled by HERC2, we measured USP16 levels upon HERC2 knockdown. As shown in Fig. 2E, HERC2 knockdown results in a reduction of USP16 protein levels (second panel, compare lanes 1 with 4) as well as an increase of H2A ubiquitination level (fourth panel, compare lane 1 with 4) as observed in previous studies (Fig. 2A). After DNA damage, the levels of HERC2 did not change significantly; however, there is a significant increase of both USP16 and H2A ubiquitination levels in control cells (Fig. 2E, second and fourth panels, compare lanes 2 and 3 with 1). In contrast, in HERC2 knockdown cells, although the basal levels of H2A ubiquitination is higher than that of control cells, the increase of USP16 was largely abolished (Fig. 2E, second panel, compare lanes 5 and 6 with 4; note that to see USP16 levels after HERC2 knockdown at the 7-h time point post-irradiation, the film was exposed at different times). Consistently, there are also very slight changes of H2A ubiquitination levels after DNA damage (Fig. 2E, second and fourth panels, compare lanes 5 and 6 with 4). These experiments indicate that HERC2 is required for the dynamic change of USP16 levels after DNA damage, which may regulate H2A ubiquitination levels in response to DNA damage. How HERC2 is involved in DNA damage-induced USP16 level changes remains an open question for future studies.

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USP16 Levels Negatively Regulate DNA Damage-induced Ubiquitin Foci Formation—Previous studies reveal that HERC2 recruits RNF8 to DNA damage foci and is required for maintaining the levels of the H2A ubiquitin ligase RNF168 (22). Our studies reveal that HERC2 also interacts with USP16, which might help recruit USP16 to DNA damage foci (Fig. 1). Moreover, HERC2 is responsible for the increased USP16 levels after DNA damage (Fig. 2). Therefore, the regulation of HERC2 on RNF8 and RNF168 seems to merge with the regulation of USP16, further suggesting that HERC2 might be central to coordinating DNA damage response. Being that RNF168 catalyzes H2A Lys-13/15 ubiquitination (11), it is not known if USP16 regulates this DNA damaged-induced H2A ubiquitination directly or indirectly. To investigate how USP16 might regulate DNA damage response, we investigated whether the levels of USP16 affect the formation of DNA damage-induced ubiquitin foci. We first examined the kinetics of DNA damage-induced ubiquitin foci formation in control and USP16 knockdown cells (Fig. 3A). To allow the observation of DNA damage response in either direction (enhancement or repression), we lowered ionizing radiation dosage, which allows ~50% of cells to have >10 ubiquitin foci at the 4-h time point after DNA damage (Fig. 3A, Control Si). Despite our extensive effort, we could not observe ubiquitin foci when the E6C5 anti-ub-H2A monoclonal antibody was used. Therefore, we used the FK2 anti-Ub antibody and observed that DNA damage-induced ubiquitin foci underwent dynamic changes. The number of distinct ubiquitin foci increased rapidly after ionizing radiation, reached maximal levels at 4 h, decreased in subsequent hours, and returned to original levels at 24 h (Fig. 3A, top panel; quantified in Fig. 3B, black line). When USP16 was knocked down (Fig. 3B, see the insets), the increase in ubiquitin foci formation was greatly accelerated, and the decrease of ubiquitin foci was significantly delayed (Fig. 3A, compare the bottom and top panels; see quantification in Fig. 3B, red line). These data indicate that USP16 functions as a negative regulator during cellular response to DNA damage and, critically, is required for the H2A ubiquitin signal returning to basal levels. We also noticed that the size of ubiquitin foci increased significantly in USP16 knockdown cells (Fig. 3C), further indicating that USP16 might function to trim down DNA damage-induced ubiquitination directly.

To further confirm the role of USP16 in DNA damage-induced ubiquitin foci formation, we examined whether overexpression of USP16 affects ubiquitin foci formation. As shown in Fig. 3, D and E, in cells overexpressing USP16, as revealed by anti-FLAG immunofluorescent staining, the number of DNA damage-induced ubiquitin foci was significantly reduced as compared with control cells without USP16 overexpression (Fig. 3D, we directly identify USP16 overexpressing cells by FLAG immunofluorescence staining). Together, these studies indicate that USP16 levels negatively regulate DNA damage-induced ubiquitin foci.

Because USP16 regulates the formation of DNA damage-induced ubiquitin foci, we investigated whether USP16 affects the recruitment of downstream repair proteins. We found that USP16 knockdown cells display quick and robust recruitment of 53BP1 compared with control cells (Fig. 4A, compare top to
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bottom panels: quantified in Fig. 4B), consistent with the notion that DNA damage-induced ubiquitination facilitates the recruitment of downstream factors. The 53BP1 foci numbers also did not return to basal levels after 24 h in USP16 knockdown cells, consistent with the persistence of ubiquitin foci in USP16 knockdown cells (Fig. 3A). To further determine the role of USP16-regulated ubiquitin foci in downstream repair protein recruitment, we measured whether overexpression of USP16 affects 53BP1 recruitment. As shown in Fig. 4, C and D, overexpression of USP16, as observed by direct red fluorescent
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protein, significantly reduced 53BP1 foci formation (red fluorescent protein-positive cells always have less DNA damage-induced 53BP1 foci). These data seem to contradict with a previous report (20). However, we noticed that different doses of ionizing irradiation were used in these experiments (0.25 gray versus 3 gray). In addition, the expression level of USP16 and specific constructs used in these experiments may also contribute to the difference. Based on these studies, we conclude that USP16, when significantly overexpressed or knocked down, regulates ubiquitin foci formation and affects the recruitment of downstream repair proteins.

**USP16 Functions Downstream of RNF8 and RNF168—** Recent studies reveal several other deubiquitinases also functioning in a negative fashion during DNA damage response (17, 20, 21, 28). To determine at which stage USP16 may exert its function, we tested whether USP16 affects RNF8 and RNF168 recruitment. For this purpose we transfected Myc-RNF8 and GFP-RNF168 into U2OS cells and observed ionizing radiation-induced foci formation. As shown in Fig. 5A, ionizing radiation induces foci formation, as revealed by Myc immunofluorescent staining and direct GFP fluorescence in control cells (third panel). After USP16 knockdown, we did not observe any significant changes in the formed foci in terms of number, size, and dynamics (Fig. 5A, last row). Quantification of the formed foci revealed that both foci were not saturated (Fig. 5B). Similar results were also observed under other doses of ionizing radiation (data not shown). These data suggest that USP16 does not affect the recruitment of upstream H2A ubiquitin ligases to DNA damage-induced ubiquitin foci. Together with the following data demonstrating that USP16 deubiquitinates both H2A Lys-15 and Lys-119 ubiquitination in vitro, it is likely that USP16 may function downstream of these ubiquitin ligases and directly deubiquitinate ub-H2A.

USP16 gene is located on human chromosome 21 and is triplicated in human Down syndrome patients. Interestingly, recent studies revealed that USP16 contributes to the somatic stem cell defects in Down syndrome (29). To determine whether human Down syndrome cell lines have altered cellular response to DNA damage, we chose two megakaryocytic cell lines, CMK, a human Down syndrome cell with triplicated chromosome 21 including USP16 gene, and Mego-1, a megakaryocytic cell line with normal number of chromosome 21, for investigation. As shown in Fig. 5C, when compared with Mego-1 cells, CMK cells display a clearly reduced response to DNA damage, as reflected by the decreased ubiquitin foci at every time point after DNA damage (compare the top with the bottom panels; see Fig. 5D for quantification). These data indicate that USP16 protein levels have a direct effect on cellular response to DNA damage. Combined with the following DNA damage repair data (see Fig. 7, A and B), this study points out a critical role for USP16 in regulating cellular DNA damage response. Being that USP16 is up-regulated in human Down syndrome and down-regulated in leukemia patients and human cancers, it is possible that altered levels of USP16 affect the ability of cancer cells to repair damaged DNA, making this an interesting subject to be explored.

**USP16 Deubiquitinates DNA Damage-induced H2A Lys-15 Ubiquitination in Vitro—** Because USP16 levels negatively regulate DNA damage-induced ubiquitin foci formation, we investigated whether USP16 could directly target DNA damage-induced H2A ubiquitination for deubiquitination. Currently, it is not clear whether the E6C5 antibody can recognize ubiquitin conjugates other than ub-H2A Lys-119, for example ub-H2A Lys-15 (Fig. 2). The failure to stain DNA damage-induced ubiquitin foci with E6C5 antibody indicates that the ubiquitin foci contains other ubiquitin conjugates or that the ub-H2A Lys-119 ubiquitin conjugate was masked by other ubiquitin conjugates. To differentiate the H2A ubiquitin conjugates

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4 Z. Zhang and H. Wang, unpublished data.
induced by DNA damage, we transfected FLAG-tagged H2A and its mutant versions into 293T cells and used FLAG antibody to specifically probe different H2A ubiquitinations. As shown in Fig. 6A, DNA damage resulted in an increase of total H2A ubiquitination, as revealed by FLAG antibody targeting the wild type H2A (compare lane 2 with 1; both ub-H2A and H2A were shown; H2A served as a loading control). The increase of total H2A ubiquitination contains both H2A Lys-119 and H2A Lys-15 ubiquitination, as revealed by a Western blot assay of cells transfected with wild type H2A, H2A K13R/K15R mutant, H2A K118R/K119R mutant, and H2A K13R/K5R/K13R/K19R mutant (K4R). Cells were directly dissolved in denature buffer and used for Western blot assay or subjected to anti-FLAG immunoprecipitation under denature conditions before Western blot assay. Ionizing irradiation induces increases of both H2A Lys-13/15 and Lys-118/119 ubiquitination significantly.

As shown in Fig. 6A, DNA damage induces increases of both H2A Lys-119 and H2A Lys-15 ubiquitination significantly. H2A ub-Lys-119 is efficiently deubiquitinated by USP16 in vitro. USP16-mediated in vitro H2A deubiquitination assay with core histones and nucleosome isolated from ionizing irradiated cells expressing FLAG-tagged H2A K13R/K15R and H2A Lys-118/119. Nonspecific bands were labeled with an asterisk.

B, an ethidium bromide-stained agarose gel containing DNA fragments purified from mononucleosomes as indicated on the top of the panel. DNA ladders are loaded on each side of the gel. Western blot assay, although we could not rule out the possibility that other deubiquitinase(s) could also target H2A ub-Lys-15 or USP16 functions indirectly. Another alternative explanation is that there may be interplay between H2A ub-Lys-15 and H2A ub-Lys-119, whereas H2A ub-Lys-15 depends on H2A ub-Lys-119, as indicated from previous studies (7, 13–15). Together, these data reveal that USP16 deubiquitinates H2A ub-Lys-15 in vitro and negatively regulates DNA damage-induced ubiquitin foci.

**USP16 Negatively Regulates Cellular DNA Damage Response**—Because USP16 regulates DNA damage-induced ubiquitin foci formation and affects the recruitment of downstream repair protein, we determined whether the USP16 level regulates the DNA damage repair process and how it is related to HERC2. We measured both NHEJ and HR processes (24, 25). As shown in Fig. 7A, knockdown of HERC2 results in a significant increase of NHEJ; however, the effects were abolished when USP16 was co-expressed. Importantly, the catalytically inactive mutant USP16 only partially reduced the effects of HERC2 knockdown, suggesting that the H2A deubiquitination activity of USP16 is at least partially required for HERC2-regulated repair process. Although knockdown of HERC2 has weaker effects on HR, expression of wild type USP16, but not the catalytic mutant USP16, also significantly decreases the HR activity in HERC2 knockdown cells (Fig. 7B). These data reveal that USP16 is an important target in HERC2-regulated DNA damage response.

To determine whether USP16 in HERC2-regulated DNA damage response has any physiological consequence, we measured the survival rate of cells with knockdown of HERC2 and expression of USP16. As shown in Fig. 7C and consistent with the results from the DNA damage repair assays, HERC2 knockdown significantly increased the survival fractions in U2OS
USP16 Negatively Regulates Cellular Response to DNA damage

![A](imageA.png)

![B](imageB.png)

![C](imageC.png)

![D](imageD.png)

![E](imageE.png)

**FIGURE 7.** USP16 regulates cellular DNA damage response. A, NHEJ assays of cells transfected with siRNA and plasmid as indicated at the bottom of the panels. USP16, but not the catalytic inactive mutant, partially rescued the effects of HERC2 knockdown on cellular damaged DNA repair. Data represent the mean and S.D. from three independent experiments. Significance was calculated with Student’s t test. B, HR assays of cells transfected with siRNA and plasmid as indicated at the bottom of the panels. USP16, but not the catalytic inactive mutant, partially rescued the effects of HERC2 knockdown on cellular damaged DNA repair. Data represent the mean and S.D. from three independent experiments. Significance was calculated with Student’s t test. C, USP16 regulates cell survival. A clonogenic assay of cells transfected with indicated siRNA and plasmid is shown. A representative experiment image is shown on the top of the panel. Data represent the mean and S.D. from three independent experiments. Significance was calculated with Student’s t test. D, clonogenic assay of Mego-1 and CMK cells with knockdown of HERC2 and USP16 as indicated at the bottom of the panel after DNA damage. USP16 is partially responsible for the decreased survival rate of HERC2 knockdown cells after DNA damage. Data represent the mean and S.D. from three independent experiments. Significance was calculated with Student’s t test. E, Western blot assay of USP16 in Mego-1 and CMK cells with or without HERC2/USP16 knockdown before and after DNA damage. GAPDH was used a loading control. The relative intensity of USP16, qualified by image J program, was shown below. Antibodies used are labeled on the left side of the panel.

To further examine the physiological significance of this regulation, we determined whether human Down syndrome cells have altered DNA damage responses. Although there is no significant difference in Mego-1 cell survival in response to DNA damage with or without HERC2 knockdown (Fig. 7D, compare column 1 with 2, 3, with 4), there is a dramatic reduction in CMK cell survival after DNA damage with HERC2 knockdown (Fig. 7D, compare column 5 with 6, 7, with 8). This is consistent with the reduced DNA damage response when USP16 is over-expressed (Fig. 7C, also see USP16 levels in Fig. 7E, top panel, compare lanes 7 and 8 with 5 and 6; relative USP16 intensity was labeled below the top panel). Because chromosome 21 contains many genes, we further investigated whether USP16 contributes to the different DNA damage responses. For this purpose, we knocked down USP16 levels in CMK cells close to the level of USP16 in Mego-1 cells (Fig. 7E, compare lane 9 with lane 6). Knockdown of USP16 significantly increased cell survival rates in response to DNA damage (Fig. 7D, compare column 9 with 7 and 8 and with 3 and 4). These data reveal a potential role of USP16 in controlling the cellular response to DNA damage in human patients. These data are also consistent with the reduced DNA damage-induced ubiquitin foci formation (Fig. 5, C and D). Together, these data reveal that the levels of USP16 have a significant effect on DNA damage repair.
DISCUSSION

In response to DNA damage, cells undertake a series of responses: stop transcription, induce cell cycle arrest, repair the damaged DNA, and restore normal cellular activities after repair (30, 31). Because in eukaryotic cells DNA repair occurs in a chromatin environment, chromatin organization and activities that alter chromatin conformation are believed to be critical for the repair process. Posttranslational histone modifications including acetylation, methylation, and phosphorylation have been shown to be important for efficient DNA repair processes (30, 32). Recently, the role of histone ubiquitination, particularly H2A ubiquitination, in DNA damage repair was highlighted (33, 34). In addition to the ubiquitination of the canonical H2A Lys-119 residue, DNA damage also induces H2A ubiquitination at Lys-13/15 residue. However, it is not known how these two ubiquitination functions in DNA damage repair. Here we provide evidence that ionizing radiation induces increase of both H2A Lys-119 and Lys-13/15 ubiquitination (Fig. 6). These data, together with studies demonstrating the function of PRC1 components in DNA damage repair indicate that both ubiquitinations may participate in DNA damage repair. Interestingly, H2B ubiquitination was also implicated in DNA damage response (35). How these ubiquitination events interplay and coordinate during cellular response to DNA damage await further studies.

USP16 is a classic H2A deubiquitinase that is required for reversing ATM-dependent gene silencing induced by DNA damage, likely through ub-H2A deubiquitination, after DNA damage repair (23, 36). Whether USP16 is directly involved in the repair process was not known. Our studies here took a further step demonstrating that USP16 directly participates in the repair process. USP16 overexpression significantly reduces the number of DNA damage-induced foci (Fig. 3, D and E). USP16 knockdown not only significantly increases the number of ubiquitin foci but also increases the size of individual foci (Fig. 3, A–C). Critically, USP16 knockdown significantly delays the recovery of DNA damage-induced ubiquitin foci, indicating a role for USP16 in reversing or terminating the ubiquitin signal (Fig. 3). The functional significance of USP16 is manifested in human Down syndrome cell lines, which contains three copies of USP16 and shows an impaired ubiquitin foci formation and DNA damage response (Figs. 5, C and D, and 7D). Together, these studies establish USP16 as an important negative regulator for cellular DNA damage response.

Recently, there have been a number of deubiquitinases identified that negatively regulate DNA damage-induced ubiquitin foci. These deubiquitinases may function in different stages during DNA damage. For example, USP44 knockdown impairs the recruitment of RNF168 and thus likely affects H2A ubiquitination indirectly (20). Similarly, Trip12 and UBR5, both, regulate the levels of RNF168 proteins (28). USP3, on the other hand, when overexpressed, suppressed ubiquitin signal clearly; however, upon knockdown, has little effect on ubH2A (both the global level by Western blot and ubiquitin foci by ubH2A antibody). In contrast, USP3 has clear effects on FK2 staining, suggesting that it may target other substrates (21). OTUB1 inhibits DNA damage induced ubiquitin foci by binding to the E2 enzyme of RNF168 (17). In this study we provide evidence that USP16 could function as a deubiquitinase for both H3 Lys-13/15 and Lys-118/119 ubiquitination in vitro. Further experiments will need to determine whether USP16 directly trims down H2A K15 ubiquitination in vivo (Fig. 6, A and C). It is also possible that another deubiquitinase(s) may target H2A ub-Lys-15 directly, and the reduced ubiquitin foci we have observed are the result of interplays between H2A ub-Lys-15 and ub-Lys-119. In summary, these studies reveal that USP16 functions as a negative regulator of DNA damage response which fine-tunes DNA damage response.

USP16 interacts with HERC2 (Fig. 1). Similar to interaction with RNF8, HERC2 may help recruit USP16 to DNA damage foci. However, despite extensive efforts made, we did not observe USP16 foci after DNA damage (data not shown). This could be due to the transient interaction between USP16 and the substrate ub-H2A. In addition to recruitment, HERC2 also regulates USP16 protein levels, similar to the regulation on RNF168 (Fig. 2). How HERC2 regulates USP16 levels remains to be determined. Recent studies reveal that both HERC2 and RNF168 are subject to sumo modification (37). Whether sumoylation is involved in HERC2-regulated USP16 remains to be determined (37). Therefore, the regulation of HERC2 in cellular DNA damage response is attributed to both the ubiquitin ligase and deubiquitinase. How could HERC2 recruit both an ubiquitin ligase and deubiquitinase to DNA damage foci? This may indicate that tightly controlled and regulated ubiquitin signal pathways exist during the proper cellular repair process. Multiple layers of ubiquitin ligases and deubiquitinases are present for this purpose. The functional interactions between these components precisely control the repair process. USP16 is down-regulated in a number of cancers whether or not down-regulation of USP16 contributes to the impaired repair ability, and increased genomic instability of tumor cells remains to be determined.

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